Cultured hepatocytes of female carp (*Cyprinus carpio*) were coexposed for 4 days to 200 nM 17β-estradiol (E2), and concentration ranges of nine known Ah receptor (AhR) agonists: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), 2,3′,4,4′,5-pentachlorobiphenyl (PCB 118), β-naphthoflavone (BNF), benzo(a)pyrene (BaP), benzo(a)anthracene (BaA), diindolylmethane (DIM), 6-methyl-1,3,8-trichlorodibenzo-[f]uran (MCFD) and hexachlorobenzene (HCB). TCDD caused a greater than 100-fold induction of cytochrome P4501A (CYP1A) activity, measured as ethoxyresorufin O-deethylase (EROD), with an EC₅₀ of 6 pM. Based on EC₅₀ values, the order of potency as CYP1A inducers was TCDD > PCB 126 > BNF > BaP > BaA > PCB 118. DIM and MCFD caused a lower maximum CYP1A induction (< 9-fold), whereas HCB caused no EROD induction at concentrations up to 6 μM. TCDD, PCB 126, BNF, BaP, and DIM also caused a concentration-dependent suppression of the secretion of the yolk protein vitellogenin (Vtg), relative to E2-treated hepatocytes. Suppression of Vtg secretion was not directly correlated with EROD activity, and the anti-estrogenic effects occurred at higher concentrations than the induction of CYP1A. This indicates that the anti-estrogenicity was not caused by increased metabolism of E2 due to induction of CYP1A. Nevertheless, the order of potency of the tested compounds for suppression of Vtg secretion was comparable to the order of potency for CYP1A induction. This concurrence suggests that the anti-estrogenicity of these compounds is AhR-mediated, but does not involve CYP1A. This could be relevant for feral fish populations, as they are frequently exposed to AhR agonists, to an extent that AhR-mediated effects are observed.

Key Words: TCDD; Ah receptor; vitellogenin; CYP1A; hepatocytes; in vitro; fish; anti-estrogen.

In recent years there has been increasing attention to the effects of estrogen-mimicking compounds in the aquatic environment. Among the better-documented effects of xenoestrogens is the induction of the yolk protein precursor vitellogenin (Vtg) in male fish (Sumpter and Jobling, 1995). However, anti-estrogenic effects have also been reported in fish. Female English sole (*Parophrys vetulus*) from contaminated sites exhibited reduced gonadal recrudescence (Johnson et al., 1988). In other fish species, aryl hydrocarbon receptor (AhR) agonists like 3,3′,4,4′-tetrachlorobiphenyl (PCB 77), Aroclor 1254, and benzo(a)pyrene (BaP), inhibited secretion of Vtg or impaired gonadal development (Chen et al., 1986; Monosson et al., 1994; Thomas, 1990; Wannemacher et al., 1992). Vtg production in the liver of fish is controlled by 17β-estradiol (E2) and is crucial for oocyte maturation (Wallace, 1985). Therefore, a reduction of Vtg synthesis could result in impaired gonadal development and reduced fertility.

AhR agonists include naturally occurring substances, but also ubiquitous aquatic contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs) (Safe, 1995). Some of the most potent agonists, e.g., PCBs, PCDDs, and PCDFs, bioaccumulate in aquatic organisms because of their lipophilic attributes and their resistance to metabolism. The presence of the AhR has been established in several mammalian and fish species (Hahn et al., 1994; Safe, 1995; Stegeman and Hahn, 1994), and activation of this receptor has been related to a diverse spectrum of biochemical and toxic responses in these animals (Safe, 1990). In rodents, PCDDs and PCDFs cause inhibition of several E2-induced responses, suggesting a relationship between AhR activation and anti-estrogenicity (Safe, 1995). Experiments in human breast cancer cell lines have confirmed this relationship by demonstrating parallel structure–activity relationships for AhR binding and anti-estrogenic effects (Krishnan and Safe, 1993; Spink et al., 1994).

Effects of AhR agonists on fish suggest that these compounds may be anti-estrogenic in fish, as they are in mammals. In the present study, anti-estrogenicity was investigated using cultured hepatocytes of female carp (*Cyprinus carpio*), which can be induced in vitro to synthesize and secrete Vtg (CARP-HEP assay) (Smeets et al., 1999a). Carp hepatocytes were coexposed to E2 and AhR agonists from different compound classes and of varying potency. These compounds include 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the planar PCB 3,3′,4,4′,5-PCB (PCB 126), the mono-ortho PCB 2,3′,4,4′,5-
PCB (PCB 118), and 6-methyl-1,3,8-trichlorodibenzofuran (MCDF) (Safe, 1990; Safe, 1995). MCDF has been characterized in mammalian systems as an AhR-binding compound that is relatively more potent as an anti-estrogen than as inducer of cytochrome P4501A1 (Safe, 1995). In addition, β-naphthoflavone (BNF), hexachlorobenzene (HCB), the PAHs BaP and benzo(a)anthracene (BaA), and diindolylmethane (DIM) were tested. DIM is the more potent gastric self-condensation product of indole-3-carbinol, a constituent of cruciferous vegetables possessing anti-estrogenic as well as AhR-activating potency in mammalian systems (Beldan et al., 1991; Bradlow et al., 1991). The effects of these compounds on Vtg secretion by carp hepatocytes were compared to their potencies as inducers of the monooxygenase cytochrome P4501A1 (CYP1A), which is one of the most sensitive AhR-mediated responses in many species (Safe, 1990).

MATERIALS AND METHODS

Animals. Fish used in these experiments were genetically uniform all female, F1-hybrid progenies of common carp (Bongers et al., 1994; Komen et al., 1991). All fish were raised at 25°C in the hatchery of the research group Fish Culture and Fisheries (Wageningen Agricultural University) and transported to Utrecht 1 to 2 months prior to their use. Subsequently, fish were kept in Utrecht municipal tapwater at a constant temperature of 24°C. Female carp (22–30 cm) were approximately 1 year old and possessed gonads with oocytes in various stages of vitellogenesis.

Cell culture and exposure. Hepatocytes were isolated, cultured and exposed as described earlier (Smeets et al., 1999a). Briefly, a two-step perfusion technique was used in which a portion of the liver was perfused, first with a Ca2+- and Mg2+-free medium containing EDTA (0.145 M NaCl; 5.4 mM KCl; 5 mM EDTA; 1.1 mM KH2PO4; 12 mM NaHCO3; 3 mM NaH2PO4; 100 mM HEPES; pH 7.5), and subsequently with medium containing 0.26 mg/ml of collagenase D (Boehringer, Mannheim, Germany). The perfused liver sections were then removed, minced, and sieved through nylon mesh. Subsequently, the hepatocytes were washed three times and resuspended in culture medium at a concentration of 10^6 cells/ml.

The hepatocytes were cultured in phenol red free DMEM/F12 medium (D2906, Sigma, St. Louis, MO, USA), supplemented with 14.3 mM NaHCO3, HEPES (final concentration 20 mM), 50 mg/l gentamycin, 1 μM insulin, 10 μM hydrocortisone, 2% v/v Ultraser-SS (steroid-free) serum (Jones Chromatography, Mid Glamorgan, UK) and 2 mg/l of the protease inhibitor aprotinin (Fluka, Buchs Switzerland) at pH 7.4. Cells were seeded in 96-well tissue culture plates (Greiner, Alphen a/d Rijn, the Netherlands) at 1 × 10^3 cells/ml, 0.18 ml/well, and maintained in air at 24°C. Each concentration of a compound was tested in six wells on one plate, unless stated otherwise. After 1 day of acclimatization, the cells were exposed for 4 days to E2 and the test compounds. Toxicant-containing medium was renewed after 2 days’ exposure.

Compounds were dissolved in DMSO (final concentration 0.2% v/v) except E2 (Sigma Chemical Co., St. Louis, MO, USA), which was dissolved in ethanol. BaP was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and α- and β-naphthoflavone (ANF and BNF) (99.5%) from Janssen Chimica (Geel, Belgium). TCDD originated from Dow Chemical (Midland, USA) and PCB 126 from Schmidt BV (Amsterdam, the Netherlands). BaA and HCB were obtained from Riedel de Haén AG (Seelze, Germany) and PCB 118 from Cambridge Isotope Laboratories Inc. (Woburn, MA, USA). DIM was a gift from Ir. J. Vuik (TNO Toxicology and Nutrition Institute Zeist, the Netherlands) and MDCF was a gift from Prof. S. Safe (Texas A&M Univ., Dept. of Veterinary Physiology and Pharmacology). After 4 days’ exposure, culture medium was transferred into 96-well plates, frozen, and kept at −70°C prior to analysis of Vtg content. Remaining cell monolayers were either used immediately for determination of cell viability, or frozen and kept at −70°C prior to EROD determinations.

Cell viability, CYP1A activity, and protein. The viability of cells, exposed for 4 days to the various compounds, was assessed by measuring mitochondrial dehydrogenase activity, using MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) as a substrate (Denizot and Lang, 1986). In addition, leakage of lactate dehydrogenase (LDH) was measured, which is indicative of membrane integrity (Bergmeyer et al., 1965). CYP1A activity was measured as ethoxyresorufin O-deethylation activity (EROD) (Burke and Mayer, 1974). The determination of viability parameters and EROD activity were performed as described earlier (Smeets et al., 1999a). Protein was measured according to the method of Bradford (Bradford, 1976).

Vtg determination. The secretion of Vtg into the culture medium was quantified by means of an indirect competitive ELISA as described earlier (Smeets et al., 1999a). Ninety-six-well EIA/RIA plates were coated with diluted blood plasma of an E2-treated female carp, containing approximately 45 mg/ml of Vtg as the dominant protein. The same plasma was used in a duplicate standard dilution curve on every plate (28,125- to 7,200-fold dilutions). Medium samples were diluted 100- to 2000-fold, in order to obtain Vtg concentrations that were within the linear part of the log-transformed standard curve. Every sample was measured in duplicate at two dilutions differing by a factor of 2. The primary antibody used was a polyclonal rabbit antibody against goldfish (Carassius auratus) Vtg that cross-reacts with Vtg from other cyprinid species such as carp (Nichols et al., 1999). The secondary antibody was an alkaline phosphatase conjugated monoclonal mouse anti-rabbit IgG (clone RG-96, Sigma, St. Louis, MO, USA). 4-Methylumbelliferylphosphate was used as substrate and quantified with a fluorescence plate reader (excitation, 360 nm; emission, 460 nm).

Calculations and statistics. Dose–response curves were fitted using the dose–response or sigmoidal curve fit option of the graphics computer program Slide Write Plus for Windows version 4.0 (Advanced Graphics Software Inc., Carlsbad, Ca, USA). Curve fits of the EROD dose–response relationships were calculated using only the rising part of the curve in order to make a sigmoidal fit possible. Statistical significance (p < 0.05) was calculated using a two-way ANOVA.

RESULTS

CYP1A Induction in Carp Hepatocytes

All compounds induced EROD activity, in a concentration-dependent manner (Figs. 1–4), with the exception of HCB, which did not cause significant induction of EROD activity over a concentration range of 6 nM to 6 μM (data not shown). In order of increasing EC50 values, the most potent EROD inducers were: TCDD, PCB 126, BNF, BaP and BaA (Table 1). These compounds induced EROD activity to a maximum value between 100 and 200 pmol/mg cellular protein/min, whereas control activity was below the detection limit of 1.7 pmol/mg/min. PCB 118, MCDF, and DIM were less potent and caused maximum EROD activities between 10 and 25 pmol/mg/min. The EROD induction levels and EC50 values of these compounds also depended on the exposure time (Figs. 1–4, Table 1). TCDD, PCB 126, and BNF had a lower EC50 value after 96-h exposure than after 18 h (Table 1). In contrast, BaP, BaA, MCDF, and DIM had lower EC50 values and lowest observed effect concentrations (LOECs) after 18-h exposure.

ANTI-ESTROGENICITY IN CARP HEPATOCYTES

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than after 96 h (Figs. 2–4). To investigate the effect of E2 on TCDD-induced CYP1A induction, hepatocytes were coexposed to 6 nM TCDD and a concentration range of E2 (20 to 600 nM). No significant effect of E2 was observed on EROD induction by TCDD (data not shown).

Anti-estrogenicity of AhR Agonists

Anti-estrogenicity was measured as reduced secretion of Vtg by carp hepatocytes coexposed to the test compounds and 200 nM E2. BaA, PCB 118, HCB (not shown), and MCDF did not cause a significant reduction in vitellogenesis at the concentrations tested (Figs. 3, 4; Table 1). Only at the highest concentrations of BaA and PCB 118, was a slight downward trend in Vtg secretion observed. In contrast, the highest tested concentrations of TCDD, PCB 126, BNF, BaP, and DIM all caused a significant reduction of Vtg secretion to less than 50% of the Vtg secretion caused by 200 nM E2 (Figs. 1–3; Table 1). The anti-estrogenic potency of these compounds is expressed as the IC$_{50}$ for Vtg secretion, which is the concentration that caused 50% reduction in Vtg relative to 200 nM E2 (Table 1).

To investigate whether the anti-estrogenic effects of TCDD and BNF are additive, a single concentration of TCDD (60 pM) was combined with three concentrations of BNF (0.6, 2, and 6 μM) (Fig. 5). TCDD alone caused a 49% reduction of Vtg production relative to 200 nM E2. The three tested BNF concentrations alone caused 52 to 81% reduction of Vtg. The combination of 60 pM TCDD and these three BNF concentrations caused a significantly greater decrease in Vtg secretion than the single compounds (76 to 85%) (Fig. 5). Coaddition of BNF had no significant ($p < 0.05$) effect on EROD induction by TCDD, as EROD was already maximally induced by 60 pM TCDD (data not shown).

Four days’ exposure to TCDD, PCB 126, BaP, or BaA at the concentrations that were also used in the anti-estrogenicity experiments did not significantly ($p < 0.05$) induce Vtg secretion in carp hepatocytes (not shown). This indicates that these compounds do not have estrogenic activity.
Effects on Cell Viability

Viability of carp hepatocytes was determined by measurement of MTT activity and LDH leakage. LDH leakage was not significantly increased by exposure to E2, TCDD, PCB 126, or BNF, at any concentration. In contrast, MTT activity was significantly reduced by 21 to 29% in cells exposed to 200 nM E2, relative to solvent-treated hepatocytes (Figs. 1–4). The most potent CYP1A-inducing compounds, TCDD, PCB 126, and BNF, caused an additional significant decrease in MTT activity of maximally 33 to 43% relative to E2-treated cells (Figs. 1 and 2). The decrease of MTT activity occurred at concentrations where EROD induction was maximal. However, at the highest concentrations of TCDD, PCB 126, and BNF, MTT activity was less suppressed. BaP, BaA, and PCB 118 also caused a significant 17 to 26% decrease of MTT activity relative to E2-treated cells, but only at the highest tested concentrations of these compounds (Figs. 3 and 4). MCDF and HCB (not shown) had no significant effect on MTT activity (Fig. 4), whereas 50 μM DIM caused a significant increase in MTT activity to 101 ± 13% of the activity in hepatocytes exposed only to DMSO.

The Effects of Coexposure to TCDD and ANF

Because ANF acts as an AhR antagonist in some experimental models, it was investigated whether this compound can inhibit the anti-estrogenicity and CYP1A induction of TCDD. Therefore, carp hepatocytes were exposed to TCDD (0.06 and 6 nM) and ANF (1, 5, 25 μM) alone and in combination. ANF (25 μM) caused a ≥ 9-fold induction of EROD activity (Fig. 6). EROD induction was more pronounced after an 18-h exposure period then after 96-h exposure (Fig. 6). Twenty-five micromolar ANF also caused a significant 39% decrease in Vtg secretion relative to E2-treated hepatocytes (Fig. 7). MTT activity was not significantly influenced by exposure to ANF at these concentrations (not shown).

Coexposure of hepatocytes to ANF and TCDD enhanced the
anti-estrogenicity of TCDD, rather than cause inhibition (Fig. 7). Coexposure to ANF also caused a significant decrease of TCDD-induced EROD activity, but only at the highest concentration of ANF (25 μM) (Fig. 6). To investigate whether ANF decreased EROD activity by direct catalytic inhibition of the CYP1A enzyme, 1, 5, or 25 μM ANF were added during the EROD incubations of TCDD-induced hepatocytes. These concentrations of ANF caused significant reductions of EROD activity by 58, 89, and 96%, respectively, relative to EROD incubations without ANF (data not shown). This indicates a direct catalytic inhibition of CYP1A by ANF.

DISCUSSION

CYP1A Induction in Carp Hepatocytes

CYP1A induction is one of the most sensitive markers for activation of the AhR. In the present study, all compounds except HCB caused induction of CYP1A activity. Therefore these substances have AhR agonist properties in carp. In the case of TCDD, PCB 126, BNF, BaP, and DIM, EROD induction decreased with increasing concentration after reaching a maximum. This is not in agreement with the general theory of receptor-mediated enzyme induction, which predicts a sigmoidal dose–response relationship. Decreases of EROD induction at high concentrations of inducer have been previously observed, both in vitro and in vivo (Haasch et al., 1993; Smeets et al., 1999b). A decline of EROD activity often does not coincide with a decrease in CYP1A protein or mRNA. The effect is thought to be caused by inhibition of CYP1A due to (competitive) binding of residual compound to the catalytic site of the enzyme (Hahn et al., 1993). This phenomenon can lead to underestimation of the maximum EROD induction and EC50, and consequently to overestimation of the AhR-activating potency of a compound. Biotransformation can also influence the dose–response relationships of AhR agonists, depending on the exposure period. The LOECs and EC50 values of DIM, MCDF, BaP, and BaA were higher after 96-h exposure than after 18 h. Biotransformation may have lowered the effective concentra-

FIG. 3. EROD induction, Vtg production, and cytotoxicity in carp hepatocytes coexposed to 200 nM E2 and various concentrations of BaP or BaA. MTT-activity (■) is expressed as percentage of the activity in the solvent control. Vtg production (●) is expressed as percentage of Vtg production by 200 nM E2. EROD induction was measured after 18-h exposure (●), or 96-h exposure (○). Error bars represent SD of six or four (EROD 18 h) measurements.
tions of these compounds during the 96-h exposure period. In contrast, TCDD and PCB 126, which have a very slow biotransformation rate in many species (van den Berg et al., 1994), had lower EC50 values for EROD induction after the longer exposure period. Lipophilic compounds such as these partition into the cells. After a medium change, the effective concentrations of these compounds may therefore be higher than before the medium change. To minimize the influence of biotransformation in the CARP-HEP assay, it may be more appropriate to compare EROD-inducing potencies of compounds after an exposure time of 18 h.

TCDD, PCB 126, BNF, BaP, and BaA all caused maximum EROD activities between 100 and 200 pmol/mg/min, which suggests that these compounds were full agonists of the AhR in carp. Based on EROD EC50 values, PCB 126 had a relative potency (REP) of 0.04 to 0.08 compared to TCDD in carp hepatocytes. This REP is similar to the proposed WHO/IPCS Toxic Equivalency Factor (TEF) of 0.1 for PCB 126 in mammals, but higher than that proposed for fish (0.005) (van den Berg et al., 1998). However, the latter TEF value is based on early life stage mortality, which generally produces lower REPs than those derived from in vivo or in vitro CYP1A induction (Bols et al., 1997; Newsted et al., 1995). BaP was 12,000-fold less potent than TCDD in carp hepatocytes after 18-h exposure. This in vitro potency is in agreement with data from an in vivo study with carp in which a 40,000-fold greater concentration of BaP was required to cause an EROD induction that was comparable to that of TCDD (van der Weiden, 1993; van der Weiden et al., 1994).

The low but significant induction of EROD activity by MCDF and DIM in carp hepatocytes could indicate that these compounds are partial AhR agonists. Mammalian studies have shown that these compounds can be both agonists and antagonists of the AhR (Chen et al., 1996; Safe, 1995). In rats, MCDF has a moderate to high binding affinity for the AhR, but is 1.5 \times 10^3-fold less potent than TCDD as EROD inducer. In addition, MCDF partly inhibits CYP1A1 induction by TCDD (Astrom and Safe, 1988; Astrob et al., 1988). Similarly, DIM can cause CYP1A1 induction in rats and bind to the AhR with an affinity 25,000-fold less than that of TCDD (Jellinck et
However, in human breast cancer cells, DIM inhibited TCDD-induced CYP1A activity and mRNA (Chen et al., 1996).

ANF caused a low induction of EROD activity in carp hepatocytes, indicating that ANF is a partial AhR agonist in carp, similar to MCDF and DIM. ANF also partly inhibited TCDD-induced EROD activity (Fig. 6). As we have shown, ANF can cause direct catalytic inhibition of CYP1A in carp. However, ANF may also have an inhibiting effect on AhR activation by TCDD. In rats and human breast cancer cells, ANF is a (partial) AhR antagonist, as it binds the AhR but does not cause receptor activation (Gasiewicz and Rucci, 1991; Merchant et al., 1993).

PCB 118 did not promote a maximum induction of EROD activity over the dose range tested in this study. Based on LOECs, PCB 118 had a potency of $10^{-6}$ relative to TCDD. This low REP is in agreement with the general observation that mono-ortho PCBs have lesser AhR potency in fish (TEF $= 10^{-3}$) than in mammals (TEF $= 10^{-4}$) (van den Berg et al., 1998). HCB did not cause CYP1A induction in carp hepatocytes. Based on the LOEC of TCDD for EROD induction in this study (0.6 pM), a possible REP of less than $10^{-7}$ can be

### TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>EROD (18 h)</th>
<th>EROD (96 h)</th>
<th>Vtg (96 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ µM</td>
<td>Eₘₐₓ¹</td>
<td>IC₅₀ µM</td>
</tr>
<tr>
<td>TCDD</td>
<td>$3 \times 10^{-5}$</td>
<td>186</td>
<td>$6 \times 10^{-6}$</td>
</tr>
<tr>
<td>PCB 126</td>
<td>$4 \times 10^{-4}$</td>
<td>134</td>
<td>$1.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>BNF</td>
<td>0.09 µM</td>
<td>138 µM</td>
<td>0.07 µM</td>
</tr>
<tr>
<td>BaP</td>
<td>0.4 µM</td>
<td>90 µM</td>
<td>0.7 µM</td>
</tr>
<tr>
<td>BaA</td>
<td>0.5 µM</td>
<td>66 µM</td>
<td>2 µM</td>
</tr>
<tr>
<td>DIM</td>
<td>4 µM</td>
<td>10 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>MCDF</td>
<td>0.4 µM</td>
<td>15 µM</td>
<td>2 µM</td>
</tr>
<tr>
<td>PCB 118</td>
<td>$\geq 3$ µM</td>
<td>$\geq 26$ µM</td>
<td>$\geq 3$ µM</td>
</tr>
<tr>
<td>HCB</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Note. ND, not detected.

*a Concentration at which EROD induction was 50% of the maximum induction by this compound.

*b Maximum EROD activity induced by this compound in pmol resorufin/ng cellular protein/min.

*Concentration that caused a 50% decrease of Vtg secretion.

**FIG. 5.** Vtg production in carp hepatocytes coexposed for 4 days to 200 nM E2 + BNF or to E2 + 60 pM TCDD + BNF. Error bars represent SD of six measurements. *Significantly different ($p < 0.05$) from BNF only.

**FIG. 6.** EROD induction in carp hepatocytes coexposed for 18 or 96 h to 200 nM E2 + ANF; for 96 h to E2 + 60 pM TCDD + ANF; or to E2 + 6 nM TCDD + ANF. Error bars represent SD of six (96 h), or four (18 h) measurements. *Significantly different ($p < 0.05$) from respective control (DMSO).
calculated for HCB in carp. This is three orders of magnitude lower than the REP that has been proposed based on data from rat and chicken (van Birgelen, 1998).

**Cell viability, CYP1A Induction, and Vtg Secretion**

The most potent AhR agonists, TCDD, PCB 126, and BNF, were not cytotoxic at the tested concentrations, as evidenced by the lack of LDH leakage. The observed suppression of Vtg secretion by these compounds was therefore not caused by reduced cell viability. The mentioned compounds did cause a decrease of MTT activity. This secondary viability parameter was suppressed at concentrations of the test compounds that caused maximal EROD induction. In addition, exposure to 200 nM E2 caused a pronounced suppression of MTT activity. However, this E2-induced suppression of MTT activity is not indicative of reduced cell viability, but possibly a side effect of large-scale Vtg production in female-derived carp hepatocytes (Smeets et al., 1999a). In cultured trout hepatocytes, E2 treatment caused a lowering of glucose production relative to CO₂ production (Korsgaard and Mommsen, 1993). It is conceivable that such a change in the energy balance of the hepatocytes could result in decreased mitochondrial MTT activity. A similar relationship might exist between CYP1A induction and a decrease of MTT activity. This would explain the concurrence of decreased EROD activity and increased MTT activity at the highest concentrations of TCDD, PCB 126, and BNF. These hypotheses are also supported by the observation that 50 μM DIM, which induced only a very low CYP1A activity, caused a > 50% decrease of Vtg secretion concurrent with a 40% increase in MTT activity relative to E2-treated cells.

**CYP1A Induction, E2 Metabolism, and Anti-estrogenicity**

The anti-estrogenic effects of the tested compounds could be caused by accelerated metabolism of E2 in carp hepatocytes. In human breast cancer cells (MCF-7), the anti-estrogenic effect of dioxinlike compounds has been linked to the induction of CYP1A-mediated E2 hydrolase activities (Spink et al., 1994). However, there is strong evidence supporting nonmetabolic pathways of anti-estrogenicity of AhR agonists in MCF-7 cells (Safe, 1995). In the present study, no evidence was found for a role of CYP1A in anti-estrogenic effects. The dose–response curves of the suppression of Vtg secretion were shifted to higher concentrations compared to the dose–response curves of EROD induction. Furthermore, coexposure to the CYP1A antagonist ANF did not inhibit the anti-estrogenic effect of TCDD, but rather enhanced it. These results are consistent with the observation that CYP1A1 did not catalyze oxidative metabolism of E2 in BNF-treated scup (Stenotomus chrysops) (Snowberger and Stegeman, 1987).

Another mechanism that could possibly accelerate the metabolism of E2 is AhR-mediated induction of the phase II enzyme UDP-glucuronyltransferase (GT). Glucuronidation is an important mechanism in the hepatic elimination of E2 in fish (Forlin and Haux, 1985). However, studies in plaice have shown that the GT isoenzymes responsible for 17β-steroid conjugation are different from the main AhR-inducible GT isoenzyme (George, 1994). This, and the expected low magnitude of GT induction, make it unlikely that it is the cause of the observed decrease in Vtg secretion.

**AhR-Mediated Anti-estrogenicity**

The most potent AhR agonists, TCDD, PCB 126, BNF, and BaP had the same order of potencies as anti-estrogens and as CYP1A inducers. This supports the hypothesis that the anti-estrogenicity of these compounds is AhR-mediated. Further support is provided by the observation that the anti-estrogenic effects of TCDD and BNF were additive, which would be expected in the case of an AhR-mediated effect. The TEF concept for AhR agonists applies to two compounds tested in this study, namely the PHAHs TCDD and PCB 126. The relative potency (REP) of PCB 126 for Vtg reduction was 0.1 compared to TCDD (based on IC₅₀), which is very similar to the REP for EROD induction after 18-h exposure (0.08). The similarity of these two REPs suggests that the TEF concept may also apply to the anti-estrogenic effects of AhR agonists. This hypothesis is in agreement with previous observations in rainbow trout (Oncorhynchus mykiss) hepatocytes (Anderson et al., 1996a).

ANF is a known AhR antagonist in MCF-7 cells, inhibiting the anti-estrogenic effect of TCDD (Merchant et al., 1993). However, in carp ANF seems to be a partial AhR agonist whose anti-estrogenic effect was additive to that of TCDD, ANF, and also DIM, caused anti-estrogenicity despite the fact that their maximum EROD induction was relatively low. Safe...
and coworkers (Safe, 1995) have shown that compounds can be weak (partial) AhR agonists for CYP1A1 induction, and still be potent anti-estrogens. For example, MCDF was 300–570 times less active than TCDD as an anti-estrogen in rat, but it was more than 10^5-fold less potent as an inducer of CYP1A1 (Astroff and Safe, 1988). In this study, MCDF did not show anti-estrogenic activity, but it was only tested at concentrations as high as 4 μM, whereas ANF and DIM caused decreased Vtg levels at concentrations of 25 and 20 μM, respectively.

AhR-mediated anti-estrogenicity has been extensively investigated in rodents and human breast cancer cells, but the exact mechanism of the effect is still unclear. A number of possible mechanisms have been proposed and the mechanism may be tissue and effect specific (Safe, 1995). One of these mechanisms is the AhR-mediated induction of phase I and II enzymes involved in E2 metabolism, which has been discussed in a previous paragraph. Other possible mechanisms of AhR-mediated reduction of Vtg secretion are the following: a) binding of the activated AhR to a repressor site in the promoter region of the Vtg gene, or induced synthesis of a modulatory protein that binds this site (Krishnan et al., 1995; White and Gasiewicz, 1993; Zacharewski et al., 1994). b) Downregulation of ER levels by activated AhR, resulting in reduced activation of the Vtg gene (Chaloupka et al., 1992; Harris et al., 1990; Wang et al., 1993; Zacharewski et al., 1991). This can be caused by a repressor site in the promoter region of the ER gene (White and Gasiewicz, 1993) or by inhibition of ER synthesis at the post-transcriptional level (Wang et al., 1993). c) Inhibited binding of E2 to the ER, either by direct interaction with activated AhR, or by AhR-mediated induction of a gene product that inhibits formation of the E2-ER complex. Alternatively, the activated AhR may inhibit binding of the E2-ER complex to an estrogen-responsive element in the Vtg promoter region. The latter suggestion is supported by results from a study with MCF-7 cells that found that activated ER and AhR mutually inhibit the DNA-binding capacity of the receptor complexes (Kharat and Saatcioglu, 1996). Our studies in carp hepatocytes have demonstrated that E2 does not influence CYP1A induction in this system. Therefore, they give no support for a mechanism of mutual inhibition of the ER and AhR (Smeets et al., 1999a).

Possible Implications for Feral Fish Populations

Induction of CYP1A is regularly observed in fish from contaminated areas. It has been used as a biomarker for exposure of fish to PAHs and PHAHs (Goksoyr and Forlin, 1992). Results presented in this study indicate that CYP1A induction could coincide with a decrease in Vtg production in female fish, confirming previous results in rainbow trout hepatocytes (Anderson et al., 1996a). Few studies have investigated the effects of AhR-activating substances on vitellogenesis in vivo. In juvenile rainbow trout, Aroclor 1254 caused a decrease in E2-induced Vtg levels (Chen et al., 1986), whereas in Atlantic croakers (Micropogonias undulatus), Aroclor 1254 and benz[a]pyrene (BaP) caused a reduction of Vtg levels during the vitellogenic period (Thomus, 1990). However, in rainbow trout exposed simultaneously to E2 and BNF, Vtg levels both increased and decreased, compared to E2-treated fish, depending on the dose levels (Anderson et al., 1996b). In the latter study, no dose–response relationship was determined for CYP1A induction by BNF, therefore, the effects on Vtg levels cannot be directly compared to CYP1A induction. In carp hepatocytes, the anti-estrogenic effects of all the tested compounds occurred only at concentrations at which EROD induction was maximal. Whether this difference between the dose–response relationships of these two effects will also occur in vivo and in other fish species needs to be investigated further.

Vtg synthesis is essential for oocyte maturation (Wallace, 1985), hence a reduction of Vtg secretion due to dioxinlike compounds could impair gonadal development in fish and consequently reproduction. This adverse effect has been shown in several in vivo studies. In zebrafish (Brachydanio rerio) TCDD caused a dose-related reduction of egg numbers and severely impaired development of previtellogenic and vitellogenic oocytes (Wannemacher et al., 1992). In female white perch (Morone americana) the planar PCB 3,3',4,4'-tetrachlorobiphenyl (PCB 77) impaired gonadal maturation (Monosson et al., 1994). Moreover, field studies with white sucker (Catostomus commersoni) and English sole (Parophrys vetulus) have found that CYP1A induction accompanied impaired gonadal development (Johnson et al., 1988; Munkittrick et al., 1994). These adverse effects could be explained by AhR-mediated inhibition of Vtg secretion by the liver. In addition, both the ER and AhR are present in various tissues and at different life stages. Therefore, anti-estrogenic effects of AhR-agonists in extrahepatic tissues could be equally important or more important than a reduction of Vtg secretion by the liver.

In conclusion, the results of this study have demonstrated that CYP1A-inducing compounds can suppress the E2-induced secretion of Vtg by carp hepatocytes. This anti-estrogenic effect was not caused by the induction of CYP1A activity. Nevertheless, the concurrence between the anti-estrogenicity and the CYP1A-inducing potency of the compounds suggests that the suppression of Vtg secretion was AhR mediated. These results stress the importance of anti-estrogenic effects in the aquatic environment next to the more frequently investigated estrogenic effects.

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